Use found in the intracellular region. the

Use of a Luciferase assay and a SandwichELISA to observe the effect of increasing Bradykinin concentration and HOE-140inhibitor use on luminescence levels and production of Interleukin-6 Probir Debnath Aims: The aim of practical 1 and 2 is to examine the effect ofbradykinin receptor inhibitor (HOE-140) on the activation of the receptor. Wewill determine this by using a luciferase reporter assay on smooth human musclecells. We will use two samples, one exposed to bradykinin and the other exposedto bradykinin with the inhibitor. These will then be incubated for 24 hours andthe luminescence levels will be measured to determine the level of activationof the receptors. In practical 3 a sandwich ELISA will be performed on thesamples obtained from practicals 1 and 2, the aim is to determine the level ofinterleukin-6 production stimulated by bradykinin. Hypothesis: Practicals 1 and 2: as the concentration of bradykininincreases, the level of bioluminescence will increase and the samples with theinhibitor will have lower levels than the sample without the inhibitor.

Practical 3: the level of IL-6 production will be highest inthe samples with the greater concentrations of bradykinin, the inhibitorsamples will have lower levels but will still show a similar trend. Introduction: G protein coupled receptors G protein-coupled receptors (GPCR) are one of the largest andmost diverse groups of receptors found on the cell surface membrane ofeukaryotes. They function by transducing external signals, which come in manyforms, such as peptides, lipids, sugars and light energy. Once the receptor isactivated, they then activate second messengers which go on to start asignalling cascade, for example, cyclic AMP is the second messenger which isinvolved in the adenylyl cyclase pathway. GPCR’s are involved in a

vast amountof function in the human body and is a very common pharmacological target formedicine. The structure of the GPCR consists of a single, globular polypeptidechain which spans the cell membrane to form seven alpha helices in thetransmembrane region, and 3 loops both in the extracellular region and in theintracellular region. An N-terminus is found in the extracellular region and aC-terminus is found in the intracellular region. The extracellular loops formpockets for the signals to bind to.

The way in which GPCR's function is theyinteract with G proteins. G proteins have an ability to bind to nucleotidesguanosine triphosphate (GTP) and guanosine diphosphate (GDP). They areheterotrimeric as they are made up of three subunits: an alpha, beta and gammasubunit. When the receptor is activated, a conformational change occurs, thisactivates the G protein. GTP then replaces GDP on the alpha subunit whichdissociates from the beta and gamma subunits and binds to effector proteins. They are then inactivated when GDP replaces GTP on the alpha subunit, this thenreforms the heterotrimer which then reassociates with the GPCR 1.

Bradykinin and its receptors Bradykinin is classified as a pro-inflammatory mediator aswell as a regulator of specific vascular and renal functions. They arevasoactive peptides which work on specific G-protein coupled receptors calledthe B1 and B2 receptors 2. These receptors are found on the cell surface of many tissuessuch as neurones in the brain stem and smooth muscle cells 3. Bradykinin receptor activation leads to changes in the levelsof intracellular calcium ion (Ca2+) concentration, which in turnaffects several mechanisms which include phospholipase C, prostaglandins, protein kinase, phospholipase A2, and the mitogen-activated protein kinase(MAPK)

pathway. Bradykinin B2 receptors are constitutively expressedwhereas the B1 receptors are inducible and are commonly induced incases of tissue injury where cytokines and endotoxins are present. Bradykininworks in the kinin system which plays a significant role in the regulation ofblood pressure and inflammatory reactions. Furthermore, the kinin system isimportant in several clinical cases such as allergic reactions, heart diseases, Alzheimer's disease and many more 2. Luciferase reporter assay The Luciferase reporter assay is a very common technique usedto study gene expression at transcriptional level.

Luciferase is classified asan oxidative enzyme which is found in several species, the most popular beingthe fireflies. It is known for giving organisms the ability to emit light. Thechemical reaction involves a substrate called luciferin which is converted tooxyluciferin via the luciferase enzyme, light is also a product of thisreaction 4. The assay consists of several steps: firstly, a clone of thegene of interest is made upstream of the luciferase gene (in this case our geneof interest is IL-6). The resulting DNA is introduced into cells via the use ofvectors. These cells are then allowed to grow in culture and then lysed torelease the proteins. The last step is where luciferin is added to the samples andthe enzymatic activity is measured via the use of a luminometer which givesluminescence readings 4. Bradykinin synergises with other signals to produce cytokinesin the airway smooth muscle cells.

The purpose of these is to regulate theairway inflammatory response. Interleukin-6 (IL-6) is a cytokine that issynergistically produced by Bradykinin 5. Sandwich ELISA A sandwich enzyme-linked immunosorbent assay (ELISA) is abiochemical technique that uses antibodies to test the presence

of an antigen. Polyclonal antibodies are often used, these recognise the antigen at multipleepitopes which allows the antigen to be captured more fully.

The assay producesa signal in the form of a colour change and then the absorbance of light ismeasured 6. In the practical asandwich ELISA is used to detect the presence of IL-6 and how much there is indifferent concentrations of bradykinin with and without inhibitor. Method: The overall procedure was split into 3 separate practicals. Practicals 1 and 2: The first part of practical 1 is to calculate a cell count. Toachieve this, mix a fixed volume of the human smooth muscle cell suspensionwith the same volume of trypan blue dye. Pipette this mixture into ahaemocytometer slide and place the slide under a microscope. Then count thenumber of cells within the 5×5 area. In my sample, I counted 46 cells.

An equation is then used to calculate the number of cells permillilitre: From my sample, I achieved a cell count of 46 and from theequation, I obtained a cell count of 920, 000 cells per millilitre. The nextstep is to find the volume of cell suspension in which contains 30, 000 cells. My calculations: You pipette this final volume of the cell suspension into theappropriate wells, as shown in Figure 2, in a volume of 50µl DMEM. The second part of practical 1 is preparing the bradykininreceptor inhibitor. The stock inhibitor solution (HOE-140) is provided at aconcentration of 6mM. A dilution is required to prepare a 50µl volume of 10µM concentration, using DMEM, perwell. 18 wells need to be filled with this inhibitor, so you prepare a final volumeof 1000µl of thediluted solution.

Mycalculations: You make a dilution made up of 1. 67µl of the stock inhibitor solutionand 998. 3µl of DMEM. Youthen pipette 50µl of thediluted inhibitor solution into the wells, using a P200, indicated by Figure 3. The final part of practical 1 is a Bradykinin serialdilution. The stock solution is provided at a concentration of 6mM, using theDMEM you need to prepare dilutions of concentrations: 10 µM, 1µM, 100nM, 10nM, 1nM and 0(control = DMEM) into the wells as shown in Figure 4.

You prepare the 10µM dilution by making an excessamount to fill 6 wells with 50µl. 400µl would be enough. Mycalculation: You make the 10µM dilution, in an Eppendorf tube, using 0. 67µl of thebradykinin stock solution with 399. 33µl of DMEM.

For the subsequentconcentrations, you transfer a tenth of the previous solution, in this case: 10% of 400 = 40µl. Transferthis volume to the next tube of the subsequent concentration (until 1nM) andfill the tube with 400 – 40 = 360µl of DMEM. After all the serial dilutionshave been made, pipette 50µl into theinto the wells indicated by Figure 4.

The Medium well should be filled with 150µl of DMEM. You then add 100µl of DMEM to the first threecolumns, and 50µl to thelast three columns so every well has a total volume of 150µl. For the last step of practical1, you add 150µl of DMEMto wells surrounding the ones indicated in Figure2.

The 96 well plate is then covered with a lid and is then incubated at 37oCand 10% CO2 for 24 hours and then stored at -20 oC until practical2. Figure 5 shows the final 96 wellplate, indicating the composition of each of the sample wells. In the first step of practical 2, you pipette 10µl of the samples,

using a P20, frompractical 1 into another 96 well plate in the same wells as indicated by Figure 2. You then seal the plate witha transparent plastic seal.

You then perform the Luciferase assay: you placethe plate into a luminometer which automatically adds 25µl of luciferase substrate(luciferin) into each well and the light produced in the subsequent 10 secondsis detected. Practical 3: Before this practical is performed, a 96 well ELISA platecoated with Interleukin-6 (IL-6) capture antibody is prepared. Using the IL-6capture antibody stock solution of 0. 5mg/ml you make a dilution to 2µg/ml in carbonate buffer. 50µl of this solution is pipetted intowells indicated in Figure 6.

You then cover this plate with a clear seal and then incubateovernight at 4°C. Wash theELISA plate with PBS-Tween using the squirt bottle and then to each well add200µl of 4%milk in PBS. Cover the plate with a seal and incubate at room temperature forone hour. In the first step of practical 3, you prepare serialdilutions of IL-6 in PBS-tween.

Table 3indicates the volumes of stock solution and PBS-tween needed to produce eachserial dilution. You then wash the plates with PBS-tween and dry the plate byblotting it on tissue paper. Repeat the was three times. You then add 50µl of the standards and samples intriplicate wells, shown in Figure 7, you then cover the plate with a film and incubate at room temperature for 20minutes. The secondary IL-6 biotinylated detection antibody isprovided at a concentration of 200µg/ml. Dilute this to aconcentration of 0. 2µg/ml usingthe PBS-tween. You need 50µlin 60 wells so prepare 3500µl of this diluted solution.

Mycalculations: You make the dilution using 3. 5µl of secondary IL-6 (detectionantibody) stock with 3496. 5µl ofPBS-tween. Wash the plate 3 times with PBS-tween and then pipette 50µl of the diluted secondary antibodyinto the wells indicated by Figure 6, cover and incubate at room temperature for 20 minutes.

You then dilute the HRP Streptavidin conjugate 1: 1000 inPBS-tween. You do this by mixing 3. 5µl of HRPStreptavidin conjugate stock with 3496. 5µl ofPBS-tween to produce the 1: 1000 dilution.

Wash the plate 3 times with PBS-tweenand blot on tissue paper. You then pipette 50µl of the diluted HRP streptavidininto the wells indicated by Figure 6, cover and incubate the plate at room temperature for 20 minutes. A 1: 1 mixture of peroxidase substrate B and TMB peroxidasesubstrate is made. Wash the plate 3 times with PBS-tween and blot on tissuepaper, you then pipette 50µl of thesubstrate mixture into the wells indicated by Figure 6. Cover the plate and then incubate in dark for 10 minutes, check regularly for a colour change: from colourless to blue. In the last step, 50µl of 4M H2SO4is added to the wells to stop the reaction and a colour change from blue toyellow is observed.

You then read the absorbance of the plate at 450nm. ResultsPracticals 1 and 2: Practical3: Evaluation: From the standard bradykinin solutions, with and withoutinhibitor HOE-140, prepared in practical 1, I obtained luminescence readings asshown in Table 4. I converted theconcentrations into log form and calculated the standard

deviations for eachaverage luminescence reading, as shown in Table5, for each concentration of bradykinin to generate a graph with errorbars.

Graph 1 shows the relationshipbetween increasing concentrations of bradykinin with the relative lightproduced in the luciferase assay. The results clearly show that the bradykininsolutions without the inhibitor generally showed a higher level of lightproduced as to the samples with the HOE-140 inhibitor present, for example: at0. 01µM concentration, the relative lightproduced in bradykinin alone was 7. 901 and the inhibitor solution produced5. 6835. When the concentration increased to 0. 1µM, thelight produced in bradykinin alone also increased to 11. 23, this also increasedin the inhibitor solution to 9.

84. Both increased but the solution without theinhibitor produced more light than the inhibitor solution. From practical 3, I first obtained average absorbance valuesfor each standard concentration of Interleukin-6 (IL-6) solutions, which wereprepared during the practical, as shown in Table6. I then took log form of the concentrations and plot a table, as shown inTable 7, with the average absorbancevalues for each IL-6 standard, additionally with the standard deviations foreach average to produce error bars. A standard curve was then produced, asshown in Graph 2, showing therelationship between increasing concentration of IL-6 and the absorbance. Thecurve shows that as the concentration of IL-6 increases, the absorbance alsoincreases.

For example, at log concentration 0 the absorbance was 0. 2. Whenincreased to log concentration 1, the absorbance also increased to 1. 682. I then

obtained the absorbances for the bradykinin samples, with and without the HOE-140 inhibitor, as shown in Table 8. Using the standard IL-6 curve in Graph 2, I then found the corresponding concentrations of IL-6 inthe bradykinin solutions.

I then took the log of the concentration of thebradykinin solutions, shown in Table 9, and then plotted the corresponding IL-6 concentrations and the standard deviationfor each average to produce error bars. Graph3 shows the relationship between increasing concentrations of bradykinin, with and without the HOE-140 inhibitor, and IL-6 concentration. From the graph, as the concentration of bradykinin increases, the concentration of IL-6 alsoincreases, for both samples with and without the inhibitor. However, theinhibitor sample generally showed lower concentrations of IL-6 compared to thesample without the inhibitor. For example, at log bradykinin concentration -1, the log IL-6 concentration for the bradykinin solution was 0. 257, and for theinhibitor solution, it was 0. 091.

Then at log bradykinin concentration 0, thelog IL-6 concentration for the bradykinin solution was 0. 616, and for theinhibitor solution, it was 0. 32. Both increased but the inhibitor solution waslower for both.

Discussion From the results I have obtained from the experiment, I canconclude that my hypotheses were correct for all three practicals. The data from practicals 1 and 2 showed that as theconcentration of Bradykinin increased, the luminescence also increased. Thistrend was also shown in the inhibitor sample, however, the amount of lightproduced per concentration of bradykinin was lower than the sample without theinhibitor. This was

expected in my hypothesis and therefore it is correct. Thereason for this is that the HOE-140 blocks some, not all, Bradykinin receptorsand this, in turn, prevents the transcription of the luciferase enzyme. Lessluciferase means lower luminescence for the same amount of luciferase substrateas fewer luciferase enzymes are active in the inhibitor solution 7. The data obtained from practical 3 showed that as the concentrationof Bradykinin increased, the level of absorbance also increased which in turnmeant the production of IL-6 was also increasing. The inhibitor sample showedthe same trend, but levels of absorbance were generally lower than the samplewithout the inhibitor.

The reason for this was due to the HOE-140 inhibitorblocking the Bradykinin receptors which prevent the synergistic activity ofIL-6 production 7. In conclusion, I believe the data I have produced is reliableto an extent. The expected outcomes from my hypothesis were proven by the data, however, some parts of the data had errors. Graph 1 shows that the first three concentrations' error barsoverlapped. This may have been due to there not being enough trials andtherefore the average is too close to each other. To avoid this error nexttime, we could include 5 trials to reduce the chances of anomalous values.

Also, in Table 8 I highlighted ananomalous result which I did not include in the average. This may have been dueto a pipetting error as I may have pipetted the wrong concentration into thiswell and therefore the reading was higher than the other trials. To avoid thisnext time, I could label each well which has been pipetted with the correctsubstance to prevent misplacement. Furthermore, Graph 3 shows that at log (bradykinin concentration) of -2,

theinhibitor had a greater IL-6 concentration than the bradykinin solution withoutthe inhibitor. I believe this is an error which may have resulted due to anerror in pipetting. As mentioned before, this could have been avoided withlabelling of wells and more repeats to get a more accurate average. A furtherimprovement to the experimental design would have been to carry out astatistical test such as a T test.

This would have shown if the data obtainedis significant enough to be able to accept the hypotheses. References 1. O'Connor C. Introduction to Essentials of CellBiology.

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